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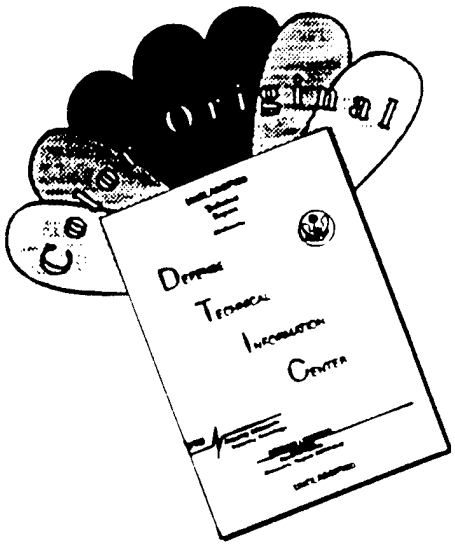

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The objective of the work is to provide a detailed understanding of the molecular mechanism by which G2/M regulation is achieved in human cells. In particular we are focusing on how initiation of M-phase is delayed in cells that have been treated with agents that induce DNA damage or that prevent synthesis of DNA. By providing a more detailed explanation of how cytotoxic therapies brings about cell death we hope to provide clinicians with better tools for the treatment of breast cancer. Human cell-lines that over-express non-phosphorylatable mutants of CDC2 were used to establish the importance of inhibitory phosphorylation of CDC2 in checkpoint control in human cells. We are currently refining these studies and developing assays for the kinases and phosphatases that regulate the phosphorylation state of CDC2 *in vivo*.

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5)Introduction

The overall objective of the work proposed in this grant is to provide a detailed understanding of the molecular mechanism by which G2/M regulation is achieved in human cells. In particular we are focusing on how initiation of M-phase is delayed in cells that have been treated with agents that induce DNA damage or that prevent synthesis of DNA. By providing a more detailed explanation of how cytotoxic therapies brings about cell death we hope to provide clinicians with better tools for the treatment of breast cancer.

6) Methodology and Results

Biochemical and cell biological techniques are being employed to address this problem. The three technical objectives that were defined in the proposed work are summarized below:

Technical Objective 1

Examine the pattern of expression and activity of mitotic checkpoint control proteins in transformed and non-transformed breast cell lines. (Equivalent to Task 1 in SOW 1-15 months)

Technical Objective 2

Examine the response of transformed and non-transformed breast cell lines to cytotoxic treatments in particular to correlate the operation of normal checkpoint controls with cell survival or death. (Equivalent to Task 2 in SOW 12-30 months)

Technical Objective 3

Determine the importance of checkpoint control proteins in cytotoxic sensitivity by genetic manipulation of WEE1, CDC25, Cyclin B and CDC2 *in vivo*.. (Equivalent to Task 3 in SOW 30-48 months)

As described in last years annual report our analysis of the pattern of expression and activity of mitotic checkpoint control proteins (CDC2, Cyclin A and B and WEE1) in transformed and non-transformed breast cell lines showed that there is over-expression of CDC2/Cyclin B in transformed breast cell-lines. However the level of over-expression is not very great and we concluded that it would not be feasible to analyse the viability of cells following cytotoxic treatment relative to these modest changes in expression level. We therefore decided to concentrate our efforts in pursuit of technical objective 3: That is to determine the importance of checkpoint control proteins in cytotoxic sensitivity by genetic manipulation of WEE1, CDC25, Cyclin B and CDC2 *in vivo*. To do this we have used the TaHela cells system of Gossen and Bujard (1) that allows conditional expression of proteins under the control of a tetracycline repressible promoter. We initiated these experiments in HeLa cells for three reasons. Firstly TAHeLa cells have been widely used; conditions for their growth and selection are already established and they provide a model system in which to test expression of particular constructs. Secondly, HeLa cells express the papillomavirus E6 protein which renders them defective for p53 function (2), therefore HeLa cells provide a model cell-line in which analysis of G2 checkpoint is not complicated by the operation of the G1 checkpoint. The data we have gained from them (see below) is likely to make an important contribution to the understanding of checkpoint control in transformed cells. Thirdly, at the time that we started the tetracycline transactivator had not been introduced into normal human breast cell-lines, but construction of such a cell-line was in progress in the laboratory of Dr. S. Reed. This cell line tTA184 now exists, and in collaboration with Dr. Reed's lab we are testing the induciblity of the cell-line and are in the process of selecting clones which express HA-CDC2TY and HA-CDC2AF (see below).

A) GENETIC MANIPULATION OF CDC2, CYCLIN B AND CDC25 *IN VIVO*

Construction of cell-lines.
Three tandemly arrayed copies of the HA eptitope were generated by PCR (3) and cloned as into pCRII (Invitrogen). CDC2, CDC2AF, CDC2A, CDC2F (4) Cyclin B, CDC25 and WEE1 were subcloned as in-frame fusions at the 3' end of the tag. The tagged constructs were sequence and subcloned into pUHD10-3, in which expression is driven by a *tet* operator fused to a minimal CMV promoter. Plasmids were co-transfected with a puramycin resistance plasmid into tTA-1 cells, a HeLa cell derivative containing the pUHD15-1neo plasmid expressing the *tet* transactivator. Clones resistant to neomycin and puramycin were screened for the inducible expression of HA-tagged CDC2, Cyclin B, CDC25 and WEE1. The use of the tag greatly facilitated analysis because it causes an apparent molecular change on SDS-PAGE such that the relative levels of CDC2 and Cyclin B can easily be judged.

Results
We first asked what happens in cell which have been delayed in S-phase due to the presence of thymidine, an inhibitor of DNA synthesis. Cells were grown in the presence or absence of tetracycline for 24 hours at which time 2mM thymidine was added to the cultures for 16 hours, the cells were washed into fresh medium and samples were taken at 0,4, 6, 8 and 10 hours after release. Nocodazole, a microtubule disrupting agent that blocks HeLa cells in M-phase, was added to the medium to prevent cells progressing beyond meta-phase. Figure 1a shows H1 kinase assays that were performed on anti-cyclin B immune-complexes prepared from these samples. As expected, control cultures, in which tetracycline repressed expression of the tagged proteins have little H1 kinase activity at the time of release from thymidine, the activity increases as the population of cells progresses towards meta-phase. No difference in the timing of activation of H1 kinase activity could be detected cells in which wild-type HA-CDC2TY is expressed. By contrast H1 kinase activity the cell-line expressing non-phosphorylatable HA-CDC2AF was already high at the zero time point when the cells were predominantly in early S-phase. There was little increase in the H1 kinase in the 10 hours following release. In order to analyse the contribution of the tagged proteins to the total H1 kinase activity extracts were first immune-depleted with anti-HA antibodies, Figure 1b, the HA depleted extracts were then immune-depleted with anti-cyclin B antibodies and the kinase assays are shown in Figure 1c. Control experiments indicate that the conditions used removed all the HA signal that could be detected by immune-blot analysis and that no activity could be measured in subsequent anti-HA immune-complexes . As can be seen in Figure 1b the anti-HA immune-precipitates show the same temporal pattern of activation as either the total cyclin B kinase activity or that attributed to the endogenous protein. Remarkably the endogenous $p34^{CDC2/Cyclin\ B}$ in cells expressing HA-CDC2TY is active at the zero time point suggesting that the activity of the non-phosphorylatable CDC2 has triggered the activation of the endogenous protein. Immune-blot analysis of these samples confirmed that the extent of phosphorylation of endogenous CDC2 is reduced in cells that express HA-CDC2AF compared to the same cell-line in which expression is repressed by addition of tetracycline, Figure 1d. Quantitation of the activity shows that the HA tagged CDC2 accounts for 20% of the total kinase activity. This estimate is in agreement with estimates of protein expression based on western analysis.

To further examine the consequences of prematurely activating CDC2 in the presence of unreplicated DNA cells were treated with thymidine as above except that cultures were allowed to progress in the absence of nocodazole. The DNA content of the cells was analyzed by propidium iodide staining and flow cytometric analysis. As shown in Figure 2A at the time of release from thymidine all the cultures have blocked with an early S-phase content of DNA. The cultures in which expression of HA-CDC2 is repressed due to the presence of tetracycline resume DNA synthesis normally, having predominately completed

synthesis 6 hours after release. An increase in G1 cells is seen 8-10 hours after release from thymidine. No significant difference was observed in the DNA content profile of cells in which wild-type HA-CDC2TY was expressed. Cell-lines that express mutant HA-CDC2AF also arrest with an S-phase content of DNA, however on release of the block a marked degeneration in the quality of the DNA profile is seen. There is a general increase in DNA content, approaching the 4N content seen in the control cultures, but there is little evidence for an orderly progression through mitosis with few cells emerging in G1 at the latter time points. Consistent with the degeneration in the quality of DNA colony survival assays of cells lines expressing HA-CDC2AF showed that ~15% of the cells survived culture in the presence of thymdine for 16 hours. To characterize more carefully the phenotype of cells following release from the thymidine block microscopic analysis was carried out on cells 6 hours following release. Staining with the DNA specific dye Hoechst 33258 and with antibodies directed to the nuclear lamina showed a significant numbers of cells contained aberrant nuclei (data not shown). The DNA appeared to be hyper-condensed and the nuclear lamina was dispersed throughout the many cells, but few cells with true mitotic figures were seen. Aberrant nuclei were present in 72% cells expressing HA-CDC2AF. The remaining 28% had normal interphase morphology. Control controls in which expression of CDC2AF was repressed by addition of tetracycline, or that expresss HACDC2TYshowed 95-98% cells had normal interphase morphology 2-5% cells had a normal mitotic morphology. The morphology of the cells expressing HA-CDC2AF suggests that the premature activation of CDC2 is not sufficient to drive cells through mitosis. An observation which is consistent with previous reports (5, 6). However the expression of HA-CDC2AF over-rides the checkpoint which normally maintains endogenous CDC2 in an inactive state and this premature activation of CDC2 has catastrophic consequences for the cell.

The same experiments were carried out using cell-lines that express HaCDC2A and HaCDC2F. Both these single mutants behaved like the wild-type construct showing no premature activation of endogenous CDC2 and no increase in cell death following exposure to thymidine. The level of expression of these constructs was equal to or greater than that of the HaCDC2AF protein. This suggests that abolition of a single phosphorylation site is not sufficient to over-come the DNA replication block. The preliminary experiments we have done with CDC25 and Cyclin B over-expression, likewise show that, expression of these proteins has no discernible phenotype. However in these cases we do not have a positive result to prove that enough protein is being produced. We have not fully tested the function of the exogenously expressed proteins and therefore more experiments are required.

Having established the importance of inhibitory phosphorylation in the checkpoint which blocks entry into M-phase in the presence of unreplicated DNA it was important to determine if the same mechanism is responsible for the G2 delay that is induced in the presence of DNA damage. We therefore examined the consequences of exposing cells to γ–radiation which induces double strand breaks in DNA. Cells were grown in the presence or absence of tetracycline for 24 hours at which time cells were exposed to 10 Gy or not treated, 6 hours later cells were harvested and extracts were prepared. The H1 kinase activity associated with tagged-CDC2 and with the endogenous CDC2/cyclin B is shown in Figure 3a. In the presence of nocodazole the untreated cells accumulate H1 kinase activity. At this dose γ-radiation there is a 25 fold reduction in the endogenous H1 kinase activity in cells which express wild type HA-CDC2TY or in which expression of HA-CDC2AF was repressed, by contrast a reduction of only 1.5-fold the level of H1 kinase was measured in cells expressing HA-CDC2AF, relative to unirradiatated cells. This experiment shows that cells expressing HA-CDC2AF continue to accumulate active CDC2 even after they have sustained damage to the DNA, analysis of the endogenous kinase activity and the HA

tagged activity Figure 3a and b, shows that the presence of non-phosphorylatable HA-CDC2AF induces activation of the endogenous kinase. Likewise in the absence of nocodazole irradaition causes a 10-fold loss of H1 kinase activity in control cells and a 3-fold loss in HA-CDC2AF cells. Similar results were obtained using lower doses of radiation (data not shown). The reduction in H1 kinase activity seen in irradiated cells expressing HA-CDC2AF may reflect the reduced rate at which these cells are passing through S-phase and accumulating Cyclin B protein (7) (and our unpublished observations). Experiments to determine the effect on cell viability are in progress. Experiments with HA-CDC2A and HA-CDC2F suggest that abolition of a single phosphorylation site is not sufficient to over-come the DNA damage checkpoint. The irradiation experiments have not yet been done on cell-lines that over-express CDC25 or Cyclin B

The loss of replication and DNA damage checkpoint control in human cells which express HA-CDC2AF suggests that the mechanism that normally prevents activation of CDC2 acts through the kinases and phosphatases that control the activity state of CDC2. Furthermore the observation that expression of HA-CDC2AF is sufficient to cause activation of the endogenous CDC2 supports models in which CDC2 is activated by an auto-feedback loop. The precise mechanism by which the checkpoint might act to interrupt the feedback loop remains to be determined. Having got these results in HeLa cells our priority over the next year will be to repeat these findings in breast cells and to develop improved assays for CDC25 and WEE1 such that we can continue with experiments designed to elucidate the details of how the DNA replication/damage checkpoint prevent activation of CDC2. These experiments were described in the original proposal . The progress we have made towards developing improved assays for CDC25 and WEE1 is described in the following section.

B) GENERATION OF SUBSTRATES FOR WEE1 AND CDC25 ASSAYS.

i) Initial experiments were carried out using 6His-Cyclin B produced by cloning the Cyclin B(8) coding sequence as an in-frame fusion in the BlueBac Vector marketed by Invitrogen. Viruses that direct the synthesis of 6His-Cyclin B were isolated. These virus were used to infect SF9 cells either alone or in combination with viruses we already had which direct synthesis of un-tagged CDC2. We were able to isolate complex both from co-infected cultures and by mixing cell lysates from cultures that had been separately infected with CDC2 and 6His-Cyclin B. However after several attempts using a number of different buffers and conditions (based on previously used conditions (9, 10) at purifying the complex we were unable to get complex pure enough to use as an assay for the relatively low levels of WEE1 that are present in human cell extracts and immune-precipitates. The major problem being back-ground phosphorylation by contaminating proteins.

ii) The second strategy we tried was to use 6-His-tagged versions of CDC2 produced in bacteria. The constructs used above for the expression of CDC2 in the TA/tet system were subcloned into the Tric-His system of Invitrogen. Cultures of 6His-CDC2TY and 6His-CDC2AF were grown to mid-log phase and IPTG was added to induce expression of the fusion proteins. A number of conditions were tried to improve the yield of soluble protein. Cells were induced in poor medium, rich medium, at 37°C and at 25°C. The soluble protein was purified on NTA-Ni agarose (Qiagen), mixed with untagged Cyclin B from baculo-infected SF9 cells and complex was isolated. Once again we found that this complex was a poor substrate for WEE1. The complex could be phosphorylated by WEE1 isolated from baculo-infected Sf9 cells, but the activity in human cell lysates and immune-precipitates could not be reliably measured with this substrate. We also tried experiments in which monomeric CDC2 was used as a substrate. Although monomeric CDC2 is known to be a relative poor substrate for WEE1 (approximately 10-fold less than

CDC2/Cyclin B (11) and our unpublished results) we reasoned that by adding relatively large quantities of denature purified substrate (protein was purified in buffer containing 8M Urea) we might be able to provide a high enough substrate concentration to favor phosphorylation. The use of denatured purification results in significantly better binding of 6His-CDC2, although there was very little background, again we were unable to assay human WEE1 activity.

iii) The strategy which we are currently using is the produce tagged-CDC2 in baculo-infected SF9 cells. The Bac-to Bac system (Gibco) was used to isolate a set of viruses that direct expression of wild-type CDC2 and the phosphorylation site mutants. These proteins have been produced in single infections or co-produced in the presence of un-tagged Cyclin B, and complex can be isolated on NTA-Ni agarose beads. We are currently working on conditions to purify these proteins. The idea is to use 6His-CDC2/Cyclin B purified under native conditions as a substrate and then if necessary clean up the product reactions by washing under denaturing conditions (e.g. 8M Urea) so that the CDC2 band can be easily identified on autoradiography. These experiments are still in progress.

We have put a lot of effort into developing an improved assay for WEE1 for two reasons. Firstly the published assays, including the ones which we have published (10, 12, 13, 14, 15), are neither simple nor sensitive and therefore might not be able to detect small, but important changes in WEE1 activity. Secondly we want a reliable, sensitive and linear assay for CDC25 activity. Despite the publication of assays for CDC25 (16) we hope that by using 32P-labeled CDC2 as a substrate we will be able to create an assay that can reliably detect small changes in CDC25 function.

7) Conclusion

The results obtained from our analysis of the over-expression of non-phosphorylatable CDC2 in HeLa cells validate the hypothesis on which much of the proposal was based. We will therefore continue and expand these experiments to include other proteins both in HeLa cells and in breast cell-lines. The results we have obtained so far indicate the likely importance of the kinases (WEE1 and family members) and the phosphatases (CDC25s) that maintain CDC2 in an inactive state in the operation of checkpoint control. Therefore we will continue with the experiment described in the original proposal that are aimed at characterizing alterations in WEE1/CDC25 in response to DNA damage and/or replication blocks.

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9) APPENDICES

Figure legends

Figure 1 Expression of HA-tagged CDC2AF induces premature activation of $p34^{CDC2/Cyclin\ B}$ in thymidine arrested cells.

A, the H1 kinase activity associated with cyclin B was measured at 0, 2, 4, 8 and 10 hours after release from thymidine in cells in which the expression of HA-CDC2TY or HA-CDC2AF was repressed or induced. B, H1 kinase activity associated with HA-tagged-CDC2 in cells in which expression of HA-CDC2TY and HA-CDC2AF is induced. C, H1 kinase activity associated with Cyclin B in extracts which have been depleted of HA-tagged protein. D, Immune-blot analysis of the phosphorylation state of endogenous CDC2 in the presence (lanes 6-10) and absence (lanes 1-5) of HA-CDCAF.

Methods
Cells were grown in the presence or absence of 2μg/ml tetracycline for 24 hours. 2mM thymidine was added for 16 hours. At zero time thymidine was removed and cells were grown in fresh medium containing 100ng/ml nocodazole. At the indicated times cells were removed from dishes by incubation in phosphate buffered saline containing 1mM EDTA and 1mM EGTA, cells were harvested by centrifugation frozen as cell pellets and stored at -70°C. Lysates were made in ice-cold lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecylsulphate (SDS) containing 2mM EDTA, 1mM Na3VnO4, 0.5mM phenylmethylsulphonyl flouride, 5μg/ml leupeptin, pepstatin and aprotinin). Lysates were cleared by centrifugation at 10,0000 xg for 10 minutes. Protein concentration of the supernatants were determined using BCA from Biorad. To measure H1 kinase activity, cell lysate (150μg) was incubated for 2 hours at 4°C with polyclonal anti-$p60^{Cyclin\ B}$ serum (8987) bound to Protein A-sepharose (Pharmacia). To measure the HA-tagged proteins 300μg of cell lysate was incubated for 2 hours with monoclonal antibody 12CA5 (provided by I.A. Wilson T.S.R.I.) coupled to protein A -sepharose. The supernatant from this immune-precipitate was then incubated with anti-Cyclin B serum. Immune-precipitates were washed three times with lysis buffer, twice with kinase assay buffer (KAB 50mM Tris pH 7.4 and 10mM MgCl2), and incubated in 50μl KAB containing 100μM ATP, 0.25μCi [γ32-ATP] (7,000 mCi/mmol; ICN). After 15 minutes at 30°C reactions were terminated by addition of SDS, products were analysed by SDS-PAGE and autoradiography. The data shown is representative of five separate experiments performed on two independently isolated HA-CDC2AF expressing clones. For immunoblots 25μg of lysate was resolved on 8-15% acrylamide-SDS gels. A polyclonal antibody against the C' terminal 12 amino-acids of human CDC2 was used, blots were developed using chemiluminescence from Pierce.

Figure 2. Expression of HA-CDC2AF impairs cell cycle progression following exposure to a DNA replication block.
Methods
The DNA content of propidium iodide stained cells was analysed by flow cytometry. Cells were cultured as above except that nocodazole was not added to the medium. At the indicated times cells were fixed in 70% ethanol, washed with PBS, incubated in the presence of 200μg/ml RNAse A for 1 hour at 37°C. Cells were stained by the addition of 40μg/ml propidium iodide and analysed on a Becton Dickinson FACSort using CellQuest software. For colony survival assays cells were plated at low density, maintained in the presence of tetracycline for one week. Colonies were then stained with Giemsa (Sigma) and counted. Triplicate plates were counted for each point analysed.

Figure 3 Expression of HA-CDC2AF increases H1 kinase activity in cells following exposure to γ-radiation.

Figure 3 Expression of HA-tagged CDC2AF induces premature activation of $p34^{CDC2/Cyclin}$ following exposure to γ-radiation.

a, the H1 kinase activity associated with Cyclin B (lanes 1,2,3,4,7,8,9 and 10) was measured in extracts that had been depleted for HA-CDC2TY protein. The H1 kinase activity associated with HA-tagged CDC2 was measured in extracts from induced cells (lanes 5,6, 11 and 12). Cells were irradiated with 10 Gy (lanes 1,3,5,7,9 and 11) or not treated (2,4,6,8,10 and 12). At the time of irradiation 100ng/ml nocodazole was added to lanes 7-12. b, same as above for a cell line expressing HA-CDC2AF. c, Immune-blot analysis of the phosphorylation state of endogenous CDC2 in the presence (lanes 3 and 4) of absence (1 and 2) of HA-CDCAF. Cells were harvetsed 6 hours after addition of 100ng/ml nocodalzole (lanes 1 and 3) or irradiation with 10Gy and addition of nocodazole (lanes 2 and 4).

METHODS: Cells were grown in the presence or absence of 2μg/ml tetracycline for 30 hours. Cells were either irradiated with 10 Gy from a ^{137}Cesium source at 3.4 Gy $min.^{-1}$ or not treated. Cells were grown for a further 6 hours either in the presence (lanes 1-6) or the absence (lanes 7-12) of 100ng/ml nocodazole. Cells lysates were prepared as above. Quantitation was on a Molecular Dynamics PhosphorImager. The data shown is representative of three separate experiments performed on two independently isolated HA-CDC2AF expressing clones.

Figure 1

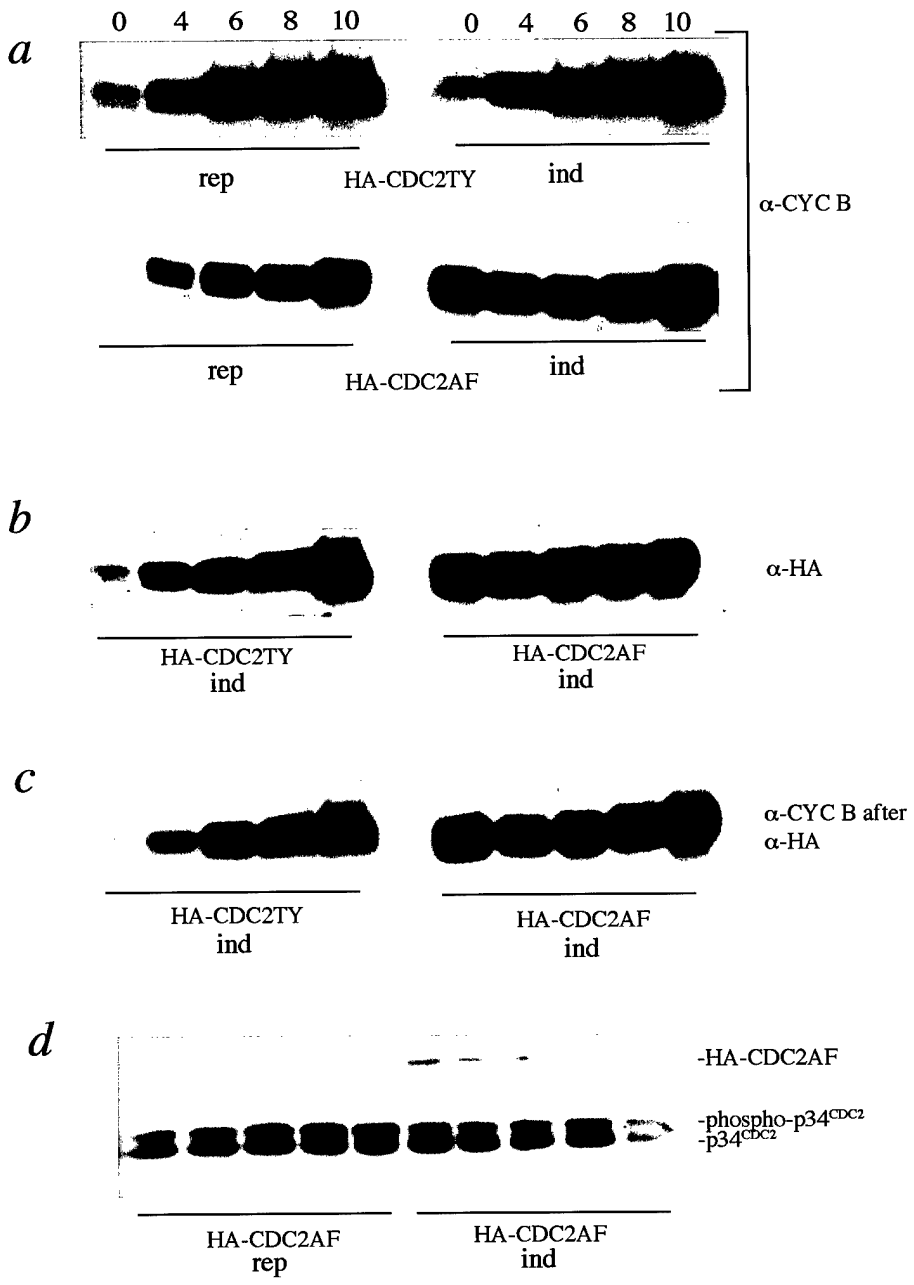
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Figure 2

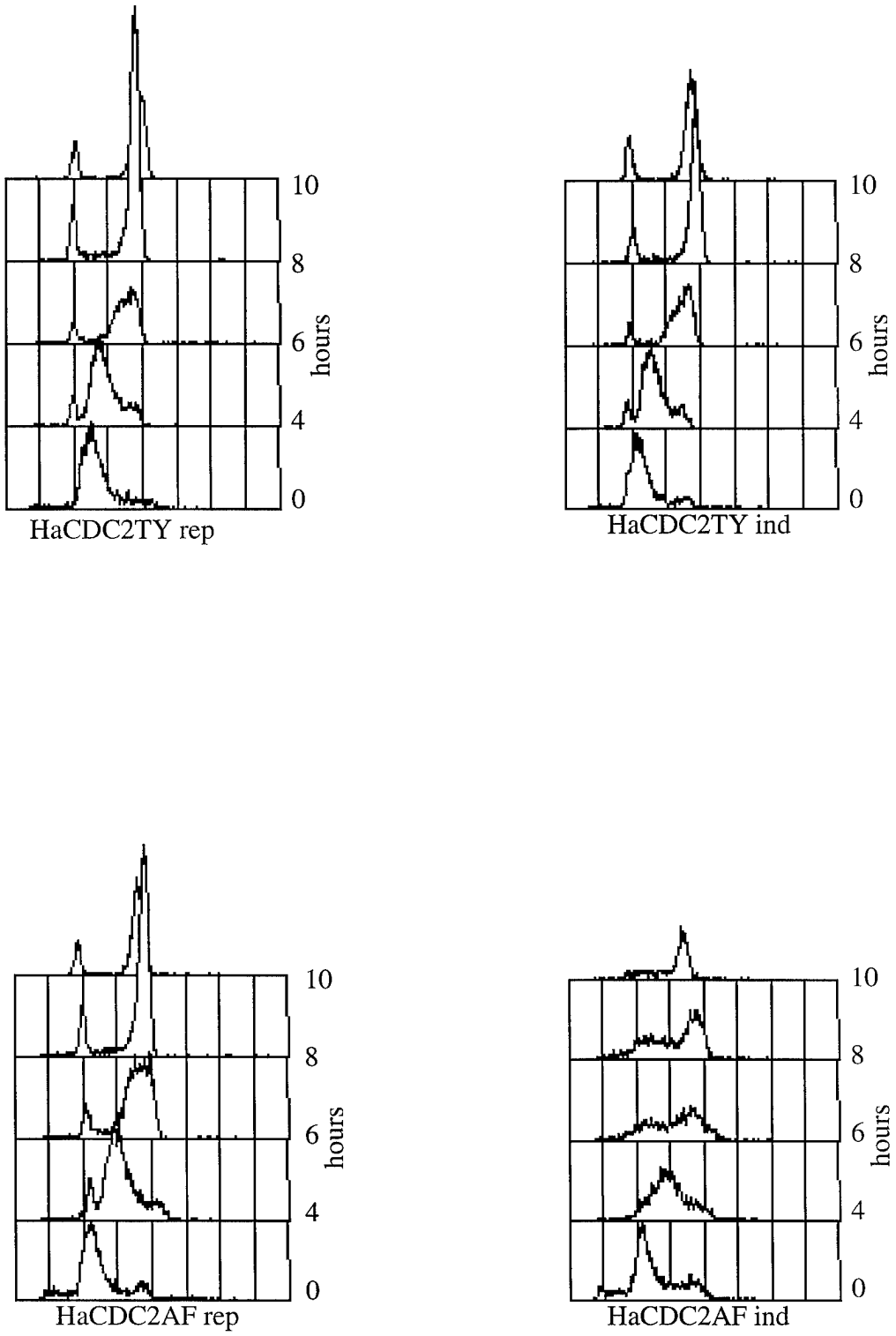
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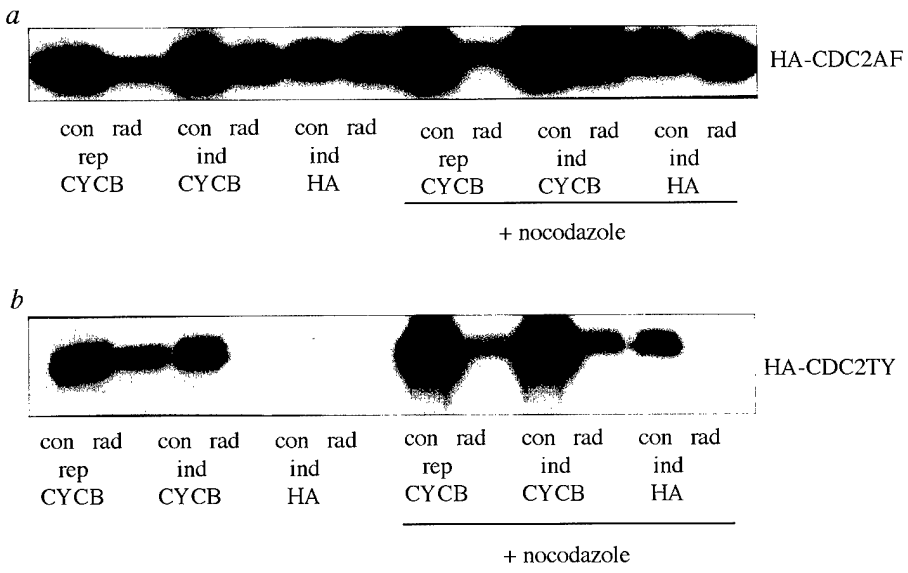
Figure 3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